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Design and synthesis of cyclic disulfide-bonded antibacterial peptides on the basis of the α helical domain of Tenecin 1, an insect defensin

Hye-sun Ahn, Wonmi Cho, Joung-min Kim, Bishnu Prasad Joshi, Jun-won Park, Chuda Raj Lohani, Hyeongjin Cho* and Keun-Hyeung Lee*

Bioorganic Chemistry Laboratory, Department of Chemistry, Inha University, 253 Younghyun-Dong, Nam-Gu, Inchon-City, 402-751, Republic of Korea

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Abstract—We synthesized cyclic disulfide-bonded (i, $i + 4$) peptides with various net positive charges (+2–+5) from linear peptides derived from the α helical domain of Tenecin 1, an insect defensin, and investigated the effect of the intradisulfide bridge (i, $i + 4$) on hydrophobicity, secondary structure, leakage activity and binding activity for large unilamellar vesicles, antimicrobial activity, and hemolytic activity. Intradisulfide bridge formation of the peptides resulted in the increase of amphiphilicity and hydrophobicity. Cyclic forms of the peptides did not deeply penetrate into PG/PC (1:1, mole ratio) large unilamellar vesicles and had a decreased lipid membrane perturbation activity for PG/PC LUVs. When the peptides interacted with PG/CL (2:1, mole ratio) LUVs, cyclic peptides with a high net positive charge (+4–+5) showed similar binding affinities and leakage activities for vesicles to those of linear forms, whereas cyclic peptides with a low net positive charge (+2–+3) exhibited lower leakage activity than their linear forms. CD spectra indicate that the intradisulfide bridge (i, $i + 4$) provided little conformational constraint to linear peptides in buffer solution but resulted in the decrease of α helicity of the peptides in lipid membrane mimic conditions. The cyclic peptide with the highest net positive charge had a similar antibacterial activity to that of the linear peptide, whereas the cyclic peptides with a low net positive charge (+3–+4) exhibited lower antibacterial activity than their linear forms. The cyclic peptides of an appropriate net charge showed more potent activities against some bacteria than those of linear forms under high salt conditions.

1. Introduction

Over the past 20 years, numerous cationic antibacterial peptides such as magainin and cecropin have been isolated from defense systems of insects, amphibians, and mammals.[1–4] These polypeptides vary considerably in chain length, hydrophobicity, and charge distribution. However, they share several common features: they are positively charged, and are able to adopt amphipathic α helical or β sheet conformations when they are associated with lipid bilayers. Most of them have a broad, but not identical, spectrum of antibacterial activity. Several structure–activity relationship studies on antibacterial peptides have revealed that important structural parameters such as amphipathicity, hydrophobicity, hydrophobic momentum, α helicity, and net positive charges all played an important role in the activity. A number of studies have demonstrated that they exert their activities by enhancing the permeability of pathogenic cell membranes.[5,6] Since the peptides have a unique mode of action, they have several features that are different from those of current antibiotics such as fast killing, bactericidal activity, a broad antimicrobial spectra, and great synergism with current antibiotics.[3–8] Therefore, they have received much attention.

Cationic antibacterial peptides have been categorized by several criteria such as structure, activity, source, and mechanism of action. Structurally, the peptides can simply be classified as linear or cyclic form. Generally, disulfide bridge(s) of proteins and peptides have been regarded as an essential requirement for activity. However, in antibacterial peptides, the requirement of disulfide bridge(s) for activity is not clear because it has been demonstrated that some antibacterial peptides did not

Keywords: Antibacterial peptide; Intradisulfide bridge; Helicity; Cyclization; Net positive charge.
* Corresponding authors. Tel.: +82 32 860 7674; fax: +82 32 867 5604; e-mail: leekh@inha.ac.kr

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lose activity upon reduction of their disulfide bridge(s). For example, the reduced linear analog of rabbit neutrophil defensin exhibited a more potent membrane-perturbation activity than the parent protein.[9] The reduction of a disulfide bridge of bactenecin resulted in an increase of activity against Gram positive bacteria and a decrease of activity against Gram negative bacteria.[10] The reduction of two intra-disulfide bridges of protegrin only decreased activity in high salt condition.[11] Furthermore, active linear fragments have been identified from cyclic host defense peptides with intra-disulfide bridges via random digestion by enzymes,[12] chemical,[13] or the random synthesis of the truncated form of the native peptides.[14–16] We also successfully extracted an active linear fragment from cyclic insect defensin protein containing three disulfide bridges, Tenecin 1.[17] The fragment corresponding to the C-terminal β sheet domain of Tenecin 1 was found to show antimicrobial activity, whereas the fragment corresponding to the α helical region of the protein did not. Previously, we synthesized several peptides with various net positive charges from the inactive α helical fragment by single or double amino acid replacement(s). We successfully identified new antibacterial peptides containing two free Cys residues, as shown in Figure 1.[18]

Recently, cyclization of peptides has been regarded as a facile method to improve stability against proteases as well as to change several structural parameters that have an effect on activity. Cyclic analogs of various linear antibacterial peptides such as melittin and magainin II were synthesized.[19–24] Most cyclic peptide analogs showed lower antibacterial activity than the parent linear peptides but some analogs exhibited improved antibacterial activity in high salt conditions.[22–24] Structure–activity relationship studies on cyclic peptide analogs of linear antibacterial peptides revealed important structural parameters for antibacterial activity.[19–22] However, cyclic intradisulfide-bonded (i, $i+4$) antibacterial peptides have not been synthesized and the effect of this short disulfide bridge on the activity has not been investigated. In the present study, we chose linear peptides derived from the α helical domain of Tenecin 1 and synthesized cyclic disulfide-bonded (i, $i+4$) peptides, as shown in Figure 1. We investigated the effect of an intradisulfide bridge (i, $i+4$) on the various structural parameters and discuss the effect of the intradisulfide bridge (i, $i+4$) on hydrophobicity, amphiphilicity, secondary structure, leakage activity for large unilamellar vesicles, and antibacterial activity.

2. Results

2.1. Design and synthesis of cyclic peptides with an intradisulfide bridge

Table 1 displays the primary amino acid sequences of several cyclic and linear peptides synthesized for this study. Linear peptides were synthesized by solid phase peptide synthesis using Fmoc-chemistry (Scheme 1).[25] After cleavage of the product from the resin, the crude peptide was purified by semi-preparative HPLC on a Vydac C_{18} column using a water (containing 0.1% TFA)-acetonitrile (containing 0.1% TFA) gradient. The successful synthesis and the homogeneity of the peptide (94%) were confirmed by analytical HPLC and MALDI-TOF mass spectrometry. As shown in Figure 2, all linear peptides derived from the α helical domain of Tenecin 1 may have an amphiphilic structure when associated with lipid membranes as analyzed by the α helical wheel diagram. We synthesized cyclic peptides (with an intradisulfide bridge (i, $i+4$)) from linear peptides by oxidation with DMSO[26] and then purified them by semi-preparative HPLC. The reduced and oxidized states of the peptides were confirmed by using both MALDI TOF mass spectrometer and Ellman assay.[27]

As shown in Table 1, the linear and cyclic forms had different retention times on our reverse phase HPLC system. The retention time, which reflects the hydrophobic interactions between the peptides and the C_{18} stationary phase, has been reported to correspond to the hydrophobicity of peptides which had biological activities related to amphipathic α helical structures.[28,29] Cyclization of all the peptides resulted in an increase of the hydrophobicity.

2.2. Interaction of peptides with large unilamellar vesicles

The membrane permeabilizing ability of the peptides was investigated by entrapped dye release from large unilamellar vesicles (LUVs). The lipid membranes of *S. aureus* mainly consist of negatively charged lipids

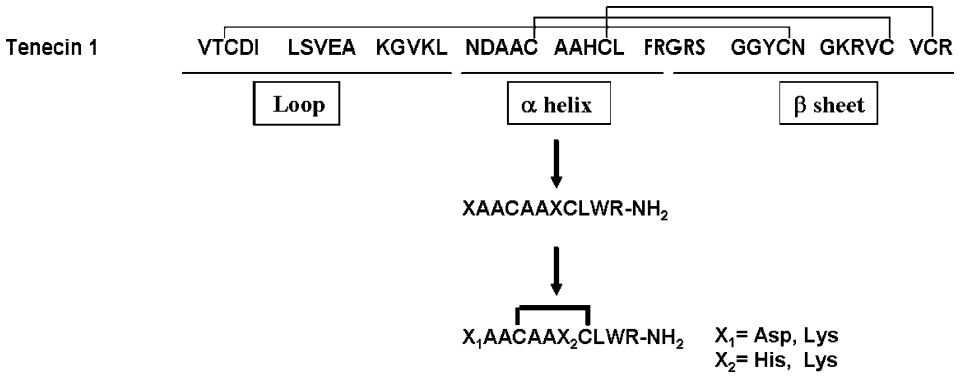


Figure 1. Design of cyclic peptides derived from the α helical domain of Tenecin 1.

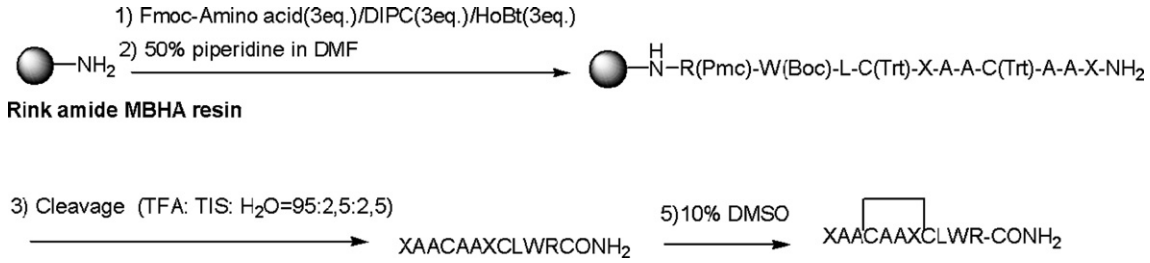
Table 1. Sequences, net charge, retention time of peptides

Name	Sequence	Net positive charge	Retention time (%)[a]	α Helicity[b]
DHWR	DAACAAHCLWR-NH_2	2	36.0	30.4
DHWRox	DAACAAHCLWR-NH_2	2	36.6	18.9
DKWR	DAACAAKCLWR-NH_2	3	36.4	26.6
DKWRox	DAACAAKCLWR-NH_2	3	37.0	15.4
KHWR	KAACAAHCLWR-NH_2	4	34.3	24.0
KHWRox	KAACAAHCLWR-NH_2	4	36.6	15.7
KKWR	KAACAAKCLWR-NH_2	5	33.8	23.1
KKWRox	KAACAAKCLWR-NH_2	5	33.9	18.7

Solvent A was water containing 0.1% (v/v) TFA and solvent B was acetonitrile containing 0.1% (v/v) TFA. The peptides were analyzed with a linear gradient of 5–50% B over 45 min.

[a] The elution of peptide was monitored by measuring absorbance at 214 nm.

[b] The α-helicity was calculated from [θ] at 222 nm measured in the presence of SDS micelles as described.[41]



Scheme 1. Synthesis scheme for linear and cyclic peptides.

such as phosphatidylglycerol (PG) and cardiolipin (CL). CL is regarded as a key lipid for the permeabilization of Gram positive bacterial cell membranes by rabbit defensins.[9] The outer lipid membranes of *Escherichia coli* contain uncharged lipids such as phosphatidylethanolamine (PE).[30] Thus, we prepared LUVs consisting of PG/CL (2:1, mole ratio) with fully negatively charged surfaces, LUVs consisting of PG/PC (1:1, mole ratio) with partially negatively charged surfaces. Various concentrations of peptides were mixed with LUVs containing fluorescent dye and then the release of entrapped dye was measured as described in the experimental section. As shown in Figure 3, KKWRox and KKWR showed similar leakage activities with vesicles. KHWR and KHWRox had slightly different leakage activities at low concentrations, however, the maximum leakage activities of the peptides were similar. In the case of DKWR and DHWR, the cyclic forms of the peptides induced relatively low release of dye in comparison to the linear form. DHWRox exhibited the lowest leakage activity. The binding of the peptides to PG/CL LUVs was investigated by using emission spectrum shift of the Trp residue, as shown in Figure 4. As the concentration of the vesicles increased, the Trp emission maxima of both the linear and cyclic forms of KKWR and KHWR were shifted to lower wavelengths (330–335 nm). This indicates that when the peptides interact

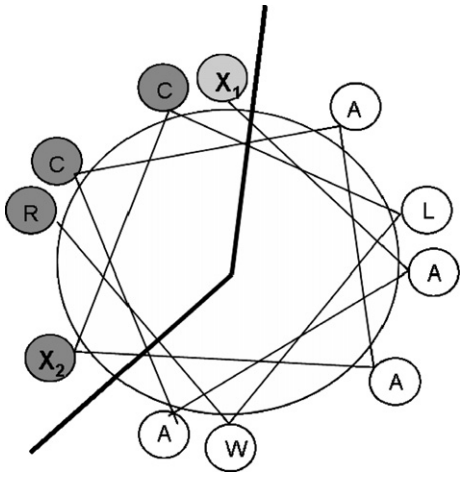


Figure 2. Helical wheel diagram of peptides.

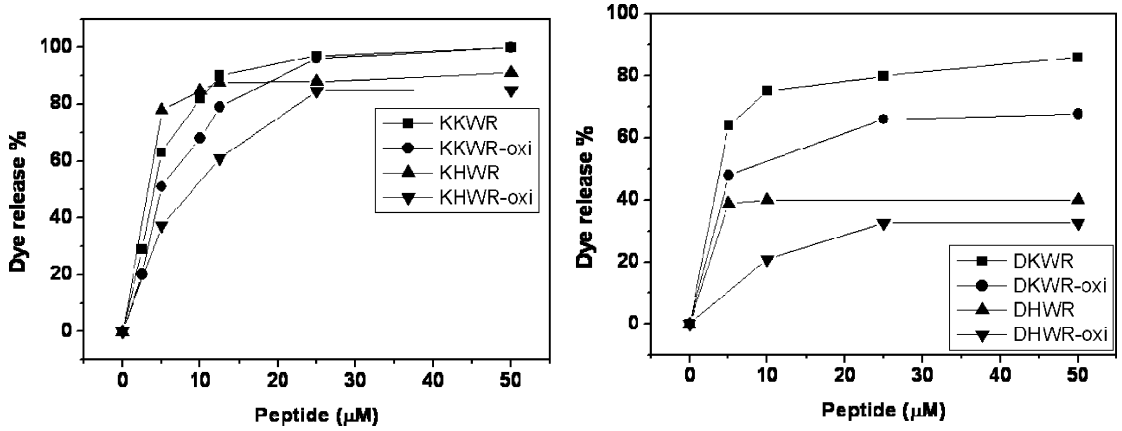


Figure 3. Leakage percent from LUVs consisting of PG/CL (2:1, mole ratio). Peptide was added into a solution of 40 μM LUVs at 22 °C and the maximum level of fluorescence, scaled to a value of 100%, was determined by complete lysis of the vesicle with TX-100. The zero level corresponds to vesicle fluorescence in the absence of peptide.

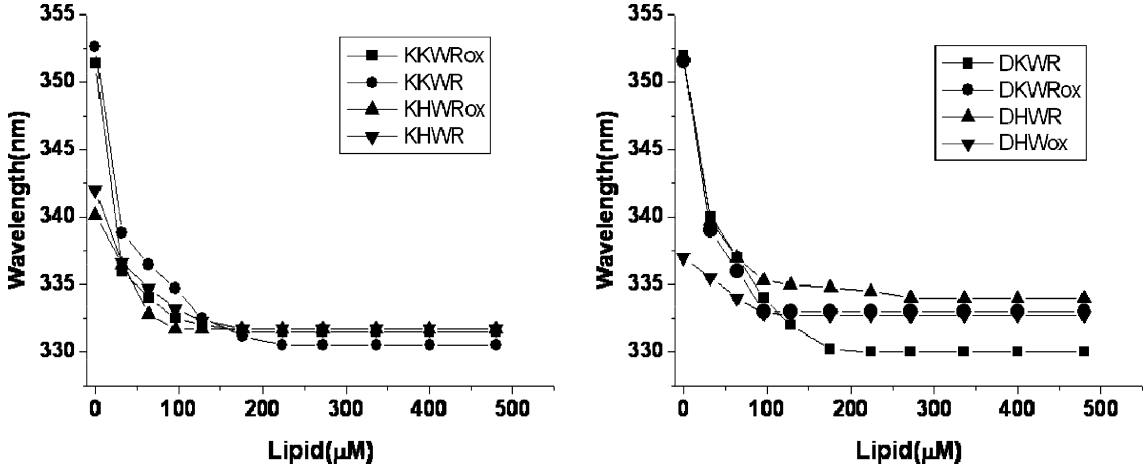


Figure 4. Maximum emission shift of peptides in the presence of PG/CL (2:1, mole ratio) LUVs. The peptide concentration was 5 μM. The maximum wavelength of emission spectrum excited with 280 nm was measured.

with the vesicles, the peptides moved to hydrophobic environment in a similar lipid concentration. The emission maxima of DKWR and DKWRox moved to lower wavelengths in the presence of the vesicles, however, the linear form exhibited a larger blue shift, which suggests that the linear form of DKWR may penetrate more deeply into the lipid bilayer than the cyclic form. In the case of DHWR, the emission maximum of the Trp residue was dramatically changed by cyclization. The cyclic form of DHWR exhibited a large blue shift even in the absence of PG/CL LUVs, indicating that the Trp residue of the cyclic form is surrounded by hydrophobic amino acid residues. A slight shift of the emission maximum of the cyclic form was observed in the presence of the vesicles, whereas a large blue shift of the emission maximum of the linear form was observed in the presence of the vesicles.

As shown in Figure 5, we investigated leakage activities of the peptides for PG/PC (1:1, mole ratio) LUVs. KKWR and KKWRox exhibited similar maximum leakage activities, however, the linear form was slightly more potent than their cyclic form at lower concentrations. KHWR also showed a more potent leakage activity than its cyclic form; KHWR induced 70% leakage of entrapped dye at 50 μM, while KHWRox induced only about 20% leakage at the same concentration. The linear and cyclic forms of peptides DKWR and DHWR with a low net positive charge (+3 and +2, respectively) induced little release of entrapped dye from the vesicles. We also investigated binding affinities of the peptides for PG/PC LUVs by using Trp emission spectrum as shown in Figure 6. The emission maxima indicate that KKWR penetrated more deeply than its cyclic form (KKWRox). KHWR and KHWRox seemed to have similar binding affinities to the vesicles. In the case of DKWR, no blue shift of the emission spectrum was observed in the presence of the vesicles. This result suggests that either DKWR or DKWRox did not bind with the vesicles or that when the peptides were bound on the vesicles, the Trp residue of the peptides was still located in a polar environment. DHWRox showed a large blue shift of emission spectrum in buffer solution as well as in the presence of vesicles, which indicated that the Trp residue of DHWRox is surrounded by hydrophobic amino acid residues. DHWR exhibited a large blue shift of

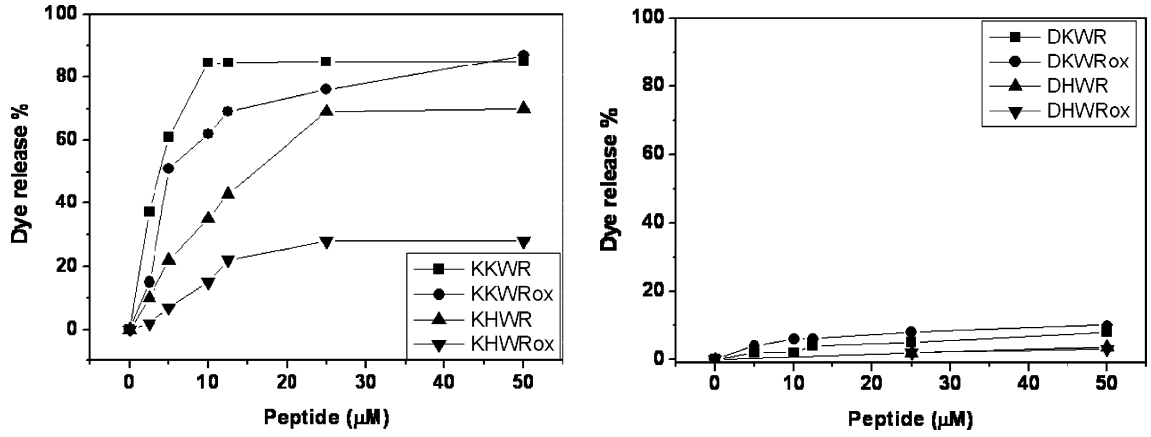


Figure 5. Leakage percent from LUVs consisting of PG/PC (1:1, mole ratio). Peptide was added into a solution of 40 μM LUVs at 22 °C and the maximum level of fluorescence, scaled to a value of 100%, was determined by complete lysis of the vesicle with TX-100. The zero level corresponds to vesicle fluorescence in the absence of peptide.

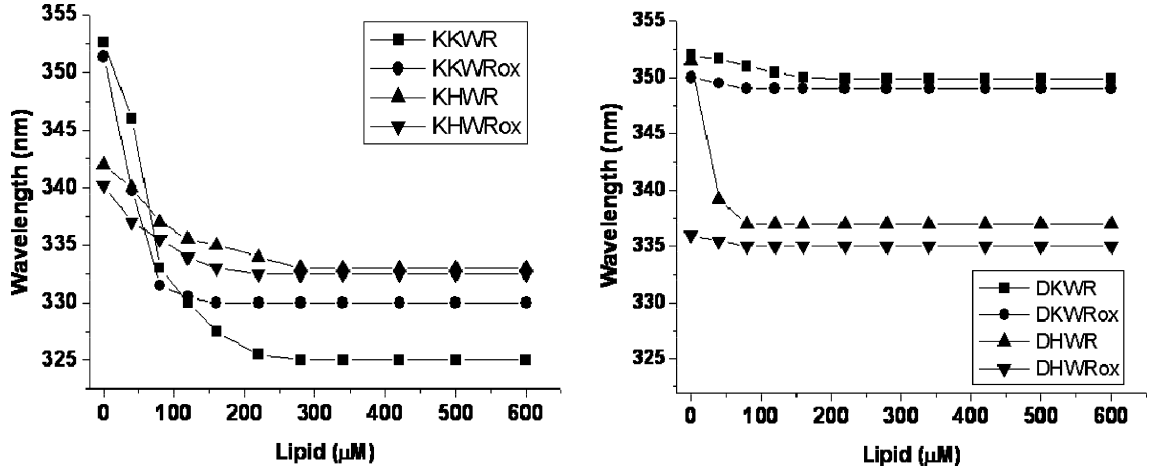


Figure 6. Maximum emission shift of peptides in the presence of PG/PC (1:1, mole ratio) LUVs. The peptide concentration was 4 μM. The maximum wavelength of emission spectrum excited with 280 nm was measured.

emission spectrum in the presence of the vesicles. This indicates that either DHWR penetrates into lipid membranes or that the Trp residue of DHWR is surrounded with hydrophobic amino acids when the secondary structure is induced during association with lipid membranes.

2.3. Antimicrobial and hemolytic activities of the peptides

As shown in Table 2, the MICs of the peptides were measured against bacteria and fungi in the absence of 150 mM NaCl. DHWR and DHWRox were inactive against bacteria and fungi at concentrations ranging

Table 2. Antimicrobial and hemolytic activities of the peptides

Peptide	Minimum inhibition concentration (μg/ml)[a]					HC_{50}[b] (μg/ml)
	M. luteus ATCC 9341	*S. aureus* ATCC 6538	*E. coli* ATCC 2592	*P. aeruginosa* ATCC 9027	*C. albicans* ATCC 36232	
DHWR	>200	>200	>200	>200	>200	>200
DHWRox	>200	>200	>200	>200	>200	>200
DKWR	200	200	200	>200	>200	>200
DKWRox	>200	>200	>200	>200	>200	>200
KHWR	25	50	50	100	>200	>200
KHWRox	100	100	>200	>200	>200	>200
KKWR	25	50	50	100	>200	>200
KKWRox	50	50	100	>200	>200	>200

[a] Average MIC values were calculated from four independent experiments performed in duplicate.
[b] The HC_{50} values were calculated by extrapolation of the fitted curve to 50% lysis of human RBCs.

from 0.8 to 200 μg/mL. Cyclization of DKWR and KHWR resulted in the decrease of activity against bacteria. The MICs of DKWR were about 200 μg/mL against bacteria but the MICs of DKWRox against all organisms were greater than 200 μg/ml. The MICs of KHWRox for various bacteria were at least 2 or 4 times less potent than those of KHWR. However, the cyclic and linear forms of KKWR (which have the highest net positive charges) have similar MIC values against bacteria.

The MICs of the peptides for bacteria and fungi were also measured in the presence of 150 mM NaCl (Table 3). These experiments were conducted because some cyclic peptide analogs of linear antibacterial peptides were reported to have more potent activity than the linear forms under high salt conditions.[22–24] Under high salt conditions, the MICs of all the peptides that had activity under low salt conditions increased. The exception to this was the activity of KHWRox which was the same under both high and low salt conditions while the MICs of KHWR increased at least 4 to 8 times. The MICs of KKWR increased 2–8 times under high salt condition as compared to those measured under low salt conditions, whereas the MICs of KKWRox increased 2–4 times. Overall, the results indicate that the intradisulfide bridge formation of the peptide with the appropriate net charge resulted in improved antibacterial activity under high salt conditions.

The hemolytic activities of the peptides were measured and are summarized in Table 2. All peptides exhibited no hemolytic activity up to 200 μg/mL. This is in contrast to a well-known hemolytic peptide, melittin, which caused the lysis of whole erythrocytes at less than 25 μg/mL [Data not shown].

2.4. CD spectra of peptides with an intradisulfide bridge

We investigated the effect of an intradisulfide bridge on the secondary structure of the peptides by using CD spectroscopy. As shown in Figure 7, the CD spectra of each peptide were measured in 10 mM Tris buffer solution (pH 7.4). The CD spectra of DHWR and DHWRox indicate that both peptides adopted a random coil as the major structure in buffer solution. The CD spectra of KHWRox and KHWR also indicate that both peptides adopted random coil structures in buffer solution.

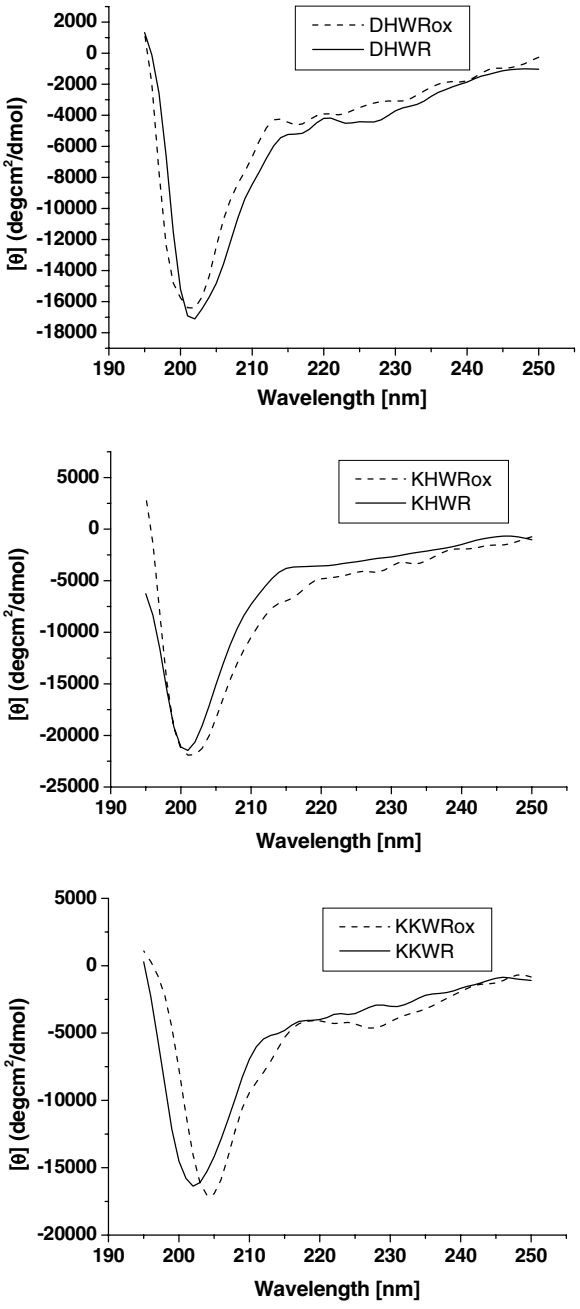


Figure 7. Circular dichrosim spectra of peptides in buffer solution. CD spectra were measured at the concentration of sample (120 μg/ml) in 10 mM Tris buffer solution (pH 7.4).

Table 3. Antimicrobial activity of the peptides in high salt condition

Peptide	Minimum inhibition concentration (μg/ml)[a]				
	M. luteus ATCC 9341	*S. aureus* ATCC 6538	*E. coli* ATCC 2592	*P. aeruginosa* ATCC 9027	*C. albicans* ATCC 36232
DHWR	>200	>200	>200	>200	>200
DHWRox	>200	>200	>200	>200	>200
DKWR	>200	>200	>200	>200	>200
DKWRox	>200	>200	>200	>200	>200
KHWR	>200	>200	>200	>200	>200
KHWRox	100	100	>200	>200	>200
KKWR	200	100	>200	>200	>200
KKWRox	200	>200	200	>200	>200

[a] Average MIC values were calculated from four independent experiments performed in duplicate.

However, the cyclic form of KKWR (KKWRox) may have a more ordered structure than its linear form as indicated by a minimum band at 205 nm in CD spectrum. The CD spectrum of DKWRox was not measured due to the poor solubility of this peptide in the buffer solution used.

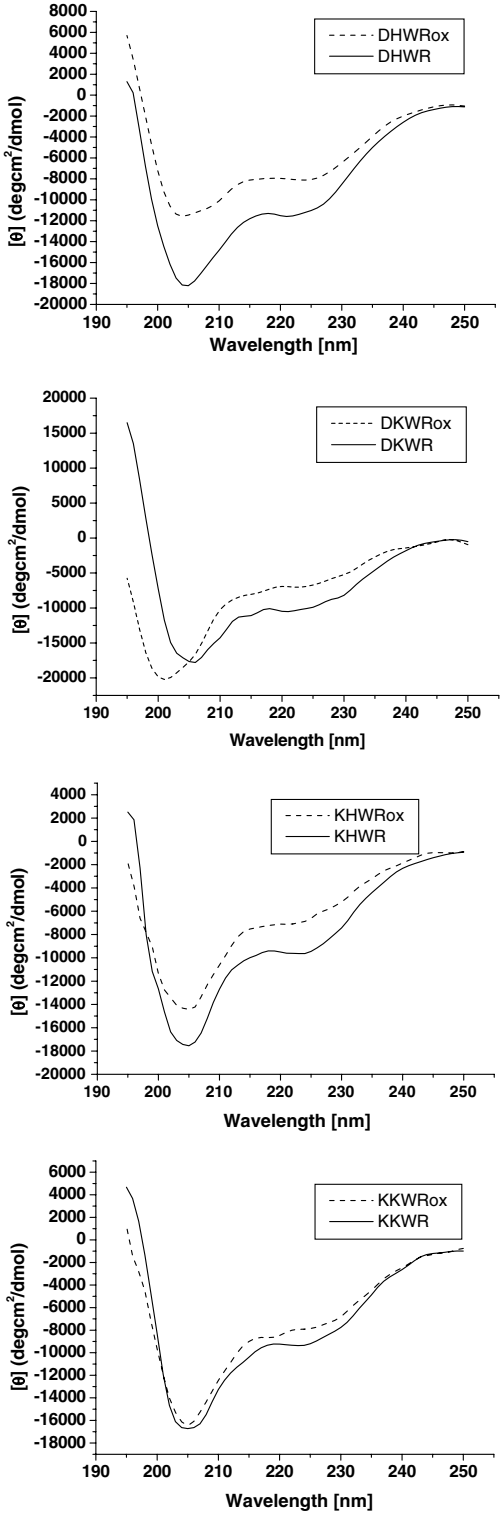


Figure 8. Circular dichrosim spectra of peptides in the presence of SDS micelles. CD spectra were measured at the concentration of sample (120 μg/ml) in 10 mM Tris buffer solution (pH 7.4) including 25 mM SDS.

It has been shown that the secondary structure of antibacterial peptides in lipid membranes, rather than in buffer solution, correlates well with antibacterial activity.[5,31,32] Since SDS (sodium dodecyl sulfate) consists of an aliphatic tail and a negatively charged head group, SDS micelles are frequently used to mimic bacterial lipid membranes.[33] As shown in Figure 8, the CD spectra of the peptides were measured in the presence of SDS micelles. DHWR and DHWRox may adopt an α helical structure based on the double minimum bands at 206 and 222 nm. However, the fractional helix content calculated by the minimum at 222 nm indicates that the α helical content of the linear form was higher than that of the cyclic form. Unexpectedly, DKWRox might adopt random structure in the presence of SDS micelles, whereas DKWR might adopt α helical structure based on the double minimum bands at 206 and 222 nm. The CD spectra of KHWR and KHWRox indicate that both peptides may adopt an α helical structure on the basis of the double minimum bands around 205 and 222 nm, however, the α helicity of KHWRox was less than that of KHWR. The CD spectrum of KKWR was similar to that of KKWRox. KKWR and KKWRox may adopt similar α helical structure upon association with lipid membranes on the basis of the minimum bands at 206 and 223 nm. The intradisulfide bridge of KKWR had no considerable effect on the secondary structure induced under conditions meant to mimic lipid membranes.

3. Discussion

In general, cyclization of peptides to generate conformationally constrained analogs has been found to play an important role in various activities of peptides. However, the requirement of cyclization of cationic antibacterial peptides for improved activity is not clear. Some cyclic antibacterial peptides exhibited improved biological activities in comparison to their linear analogs but others did not. For example, intramolecular disulfide bonds enhanced antimicrobial and lytic activities of protegrins under high salt conditions.[11,34] Replacement of all the Cys residues with α-aminobutyric acid completely abolished the chemotactic activity of human beta defensin 3, but preserved the bactericidal activity.[35] However, some of the cyclic peptides exhibited decreased antibacterial activity in comparison to the linear peptides. The cyclization effects on the structural parameters for activity have not been extensively studied.[19–21] Specifically, short cyclic disulfide-bonded (i, $i+4$) antibacterial peptides have not been synthesized and the effect of this short disulfide bridge on the structural parameters of antibacterial peptides such as hydrophobicity, amphiphilicity, and secondary structures has not been investigated. In the present study, we synthesized cyclic disulfide-bonded (i, $i+4$) peptides with positive net charges ranging from +2 to +5. These cyclic peptides were synthesized from the linear antibacterial peptides derived from the α helical domain of an insect defensin protein. The effect of the disulfide bridge on the various structural parameters and binding and leakage activity for vesicles with different surface charges was investigated. According to the reverse-phase HPLC retention

times, the cyclic forms of the peptides, except for KKWR, are more hydrophobic or amphiphilic than their linear forms. The retention time, which reflects hydrophobic interactions between peptides and the C_{18} stationary phase, has been reported to correspond to the hydrophobicity of peptides.[28,29] Our results indicate that there is a considerable effect of the disulfide bridge (i, $i + 4$) on hydrophobicity and amphiphilicity of the peptides studied herein. KKWRox, with the highest net positive charge among the peptides studied, had a similar retention time to that of KKWR, suggesting that the cyclic and linear forms of KKWR have similar hydrophobicity or amphiphilicity. Even though DHWRox had a similar retention time to that of the linear form, a large blue shift of the Trp emission maximum was observed which indicates that the Trp residue may be surrounded by hydrophobic amino acid sequences in the cyclic form. This suggests that the cyclic form had a similar hydrophobicity as compared to the linear form, but had an increased amphiphilicity.

The CD spectra revealed the effect of the intradisulfide bridge (i, $i + 4$) on the secondary structure of the peptides in both buffer solution and in lipid membrane mimic conditions. The intradisulfide bridge (i, $i + 4$) provided little conformational constraint to DHWR and KHWR in buffer solution as demonstrated by the CD spectra of the cyclic and linear forms. These spectra indicate that the linear and cyclic forms of DHWR and KHWR may adopt random coil structures in buffer solution. In contrast, the intradisulfide bridge provided some constraint to KKWR in buffer solution as indicated by the CD spectra. KKWRox may adopt a more ordered structure than that of KKWR based on the minimum bands observed at 202 and 230 nm.

We also investigated the effect of the intradisulfide bridge on the secondary structure induced under conditions designed to mimic a lipid bilayer. The CD spectra measured in the presence of SDS micelles revealed that the linear and cyclic forms of DHWR, KHWR, and KKWR may adopt α helical structures as the major structure on the basis of minimum bands observed at 205 and 222 nm. The α helicity of the peptides was calculated by the mean residue ellipticity at 222 nm, as shown in Table 1. Among the peptides, DHWR had the highest α helicity. This may be due to the charge interactions between Asp and His residues (i, $i + 6$). DHWRox and KHWRox had lower α helicity than that of their linear forms. However, the intradisulfide bridge of KKWR did not have a considerable effect on the α helicity. This may be due to the fact that KKWR adopted a more distorted α helical structure than the other peptides or that KKWR contained two Lys residues since these residues are known to have high α helical propensity among amino acids. Lactam bridges linking (i, $i + 3$) and (i, $i + 4$) spaced residues have been reported to stabilize α helical structure.[36,37] However, our result indicated that the intradisulfide bridge (i, $i + 4$) of almost all peptides employed in this study may actually destabilize α helical structure due to the short length of the intradisulfide bridge. Previously, we investigated the effect of an intradisulfide bridge (i, $i + 6$) on the structure of brevinin 1E (from *Rana esculanta*) consisting of 24 amino acid residues.[38] Even though the short length of intradisulfide bridge (i, $i + 6$) spaced residues might be expected to stabilize turn structure rather than α helical structure, the intradisulfide bridge (i, $i + 6$) retained α helical content of the linear form of brevinin 1E. The effect of an intradisulfide bridge (i, $i + 4$) of KKWR on the secondary structure might be similar to that (i, $i + 6$) of brevinin 1E.

We investigated leakage activities and binding affinities of the peptides by using LUVs with various surface charges. As shown in Figure 3, KKWR and KKWRox exhibited almost the same leakage activity for PG/CL LUVs, whereas KHWR seemed to have more potent leakage activity than its cyclic form. DKWR and DHWR definitely showed a more potent leakage activity than their cyclic forms. The leakage activity of the peptides correlated with their Trp emission maxima. Generally, a large blue shift of Trp emission maximum indicates deep penetration of the peptide into lipid membranes. The peptides that deeply penetrated into PG/CL LUVs may show high leakage activities. The emission maxima (Fig. 4) indicate that even though cyclization of DHWR and DKWR increased hydrophobicity or amphiphilicity, cyclization seemed to prevent deep penetration of the peptide into the vesicles, perhaps due to the decrease of α helicity by cyclization.

We also measured leakage activities and binding affinities of the peptides for LUVs of PG/PC (1:1), which have a partially negatively charged surface. Interestingly, the MICs of the peptides against bacteria measured under low salt conditions correlated well with leakage activity for PG/PC LUVs (Fig. 5). KKWR and KKWRox exhibited similar leakage activity for PG/PC LUVs, whereas KHWR showed more potent leakage activity than its cyclic form. The leakage activity of linear and cyclic forms of KKWR and KHWR correlated with their Trp emission maxima. Trp emission maxima shift (Fig. 6) indicates that cyclization increased binding affinities of KKWR and KHWR for the vesicles because the shift of emission maxima of cyclic forms was present at a low concentration of the vesicles. However, the cyclization prevented deep penetration of the peptides into the vesicles. Cyclic and linear forms of DHWR and DKWR did not induce the release of entrapped dye from the vesicles. The emission maxima of DKWR and DKWRox (Fig. 6) indicate that they did not bind with PG/PC LUVs because the emission maxima of the peptides did not move in the presence of the vesicles. Interestingly, DHWR exhibited a large blue shift of its emission spectrum in the presence of the vesicles, whereas DHWRox had a large blue shift of emission spectrum in buffer solution and the emission maximum did not change in the presence of the vesicles. This result indicates that the Trp residue of DHWRox was not surrounded by hydrophobic lipid membranes but by hydrophobic amino acids. DHWR had a large blue shift of emission maxima of Trp in the presence of the vesicles. This large blue shift of emission maxima of DHWR may not be due to the penetration of the peptide into lipid membranes. We hypothesized that when DHWR interacted with vesicles, the peptide adopted an α helical structure, resulting in the enveloping

of the Trp residue by hydrophobic amino acids, resulting in the large blue shift. The leakage activities of the peptides for PG/PC LUVs can be explained by considering their penetration depths into lipid membranes. Overall results indicate that the high net positive charge was a prerequisite factor for the activity and cyclization improved binding affinities, but prevented deep penetration of the peptide into PG/PC vesicles, resulting in the decrease of leakage activities.

The antibacterial activities measured under low salt conditions may correlate with the leakage activities for PG/PC LUVs. Under low salt conditions, cyclization of the peptides, except for KKWR, resulted in a decrease of the antibacterial and leakage activities. Cyclization resulted in the change of several important parameters of the activity of the peptides such as hydrophobicity, amphiphilicity, and α helicity. We supposed that the low penetration of cyclic peptides into lipid membranes is related to the decrease of α helicity of the cyclic forms. Cyclization increased hydrophobicity and amphiphilicity, resulting in the increase of binding affinity for the vesicles but decreased α helicity, resulting in the decrease of activity. We hypothesized that the optimal balance of the structural parameters of the peptides was critical for activity. If the other structural parameters of the peptides such as hydrophobicity, net charge, and amphiphilicity were satisfied, α helicity was also a critical parameter for the activity. We also investigated antimicrobial activity under high salt conditions because head to tail cyclic analogs have shown improved antibacterial activity in comparison to those of linear peptides.[21–24] The disulfide bridge (i, $i + 4$) prevented the decrease of antibacterial activity of the peptides under high salt conditions. KHWRox showed the most potent antibacterial activity among the peptide series. However, we could not explain the reason why under high salt condition, cyclic KKWR exhibited a more dramatic decrease in the activity than cyclic KHWR did.

In conclusion, we chose to synthesize peptides derived from the α helical domain of Tenecin 1 as model peptides and investigated the effect of the disulfide bridge (i, $i + 4$) on the activity and structural parameters. The intradisulfide bridge formation (i, $i + 4$) resulted in the change of several important parameters for the activity such as hydrophobicity, amphiphilicity, and α helicity. The optimal balance of the structural parameters of the peptides was critical for activity. If the other structural parameters such as hydrophobicity, net charge, and amphiphilicity were satisfied, α helicity was critical. The cyclic peptides of an appropriate net charge exhibited similar antibacterial activity under low salt conditions, however, they showed more potent activities against some bacteria than the linear forms under high salt conditions.

4. Experimental

4.1. Materials

L-α-phosphatidyl-DL-glycerol from egg yolk (PG), L-α-phosphatidylcholine from egg yolk (PC), cardiolipin from bovine heart (CL), and sodium dodecyl sulfate (SDS) were purchased from Sigma. All Fmoc amino acid derivatives, reagents, and solvents used in the peptide syntheses were purchased from Applied Biosystem. Trifluoroacetic acid (TFA), Thioanisole, calcein, and dimethyl sulfoxide (DMSO) were purchased from Aldrich Chemicals. All chemicals were of reagent grade and used without further purification.

4.2. Synthesis of peptides

All peptides tested were synthesized using Fmoc-chemistry by solid phase peptide synthesis according to the literature procedure.[25] Deprotection and cleavage were achieved by treatment with a mixture of trifluoroacetic acid (TFA)/water/thioanisole/ethendithiol (8.5/0.5/0.5/0.5, v/v/v/v) or TFA/TIS/water (95/2.5/2.5, v/v/v) at room temperature for 3–4 h. After cleavage of the product from resin, the peptides were purified by preparative-HPLC using a water (0.1% TFA)-acetonitrile (0.1% TFA) gradient (5–50% acetonitrile over 45 min). The peptides were characterized by ESI mass spectrometer (Platform II, micromass, Manchester, UK) and MALDI TOF mass spectrometer (Voyager-DE STR, Applied Biosystem). DHWR (MS: calc. 1214.53, obs. 1215.93 $[M+H]^+$), DKWR (MS: calc. 1205.56, obs. 1206.73.04 $[M+H]^+$), KHWR (MS: calc. 1227.60, obs. 1229.33 $[M+H]^+$), KKWR (MS: calc.1218.63, obs. 1219.99 $[M+H]^+$). The homogeneity (>94%) of the compound was confirmed by analytical HPLC on a C_{18} column.

4.3. Synthesis of intradisulfide bridged peptides

The linear form of peptide was diluted below 5 mM in phosphate buffer, pH 7.4, in the presence of 10% (v/v) DMSO.[26] After stirring at room temperature, the oxidation reaction was monitored according to the method of Ellman et al.[27] After completion of the oxidation, cyclic peptides were purified by HPLC on a C_{18} column. The Ellman method and MALDI-TOF mass spectrometry were used to confirm that the cyclic peptides had formed intradisulfide bridges. DHWRox (MS: calc. 1212.51, obs. 1213.89 $[M+H]^+$), DKWRox (MS: calc. 1203.54, obs. 1204.76 $[M+H]^+$), KHWRox (MS: calc. 1225.58, obs. 1227.19 $[M+H]^+$), KKWRox (MS: calc.1216.61, obs. 1217.95 $[M+H]^+$). The homogeneity (>95%) of the compound was confirmed by analytical HPLC on a C_{18} column.

4.4. Preparation of large unilamellar vesicles and leakage assay

Large unilamellar vesicles approximately 100 nm in diameter containing calcein were prepared by the freeze/thaw and extrusion method as previously described.[30,39] Lipid films were re-suspended in 10 mM tris buffer (pH 7.4) containing 70 mM calcein, 154 mM NaCl, and 0.1 mM EDTA. The size distribution of vesicles was measured by using a laser light scattering technique (Zeta sizer, Malvern). Phospholipid concentration was determined as described by Vaskovsky et al.[40] For the leakage assay, fluorescence, excited at 490 nm and emitted at 520 nm, was measured with a Perkin-Elmer

LS55 Luminescence spectrometer. The percent of dye-release caused by sample was evaluated by the equation, dye-release (%) = 100 $(F - F_o)/(F_{max} - F_o)$, where F_o was the fluorescence intensity of vesicles without peptide or TX-100, whereas F and F_{max} are intensities of the fluorescence achieved by peptide and Triton X-100, respectively.

4.5. Circular dichroism

CD spectra were recorded on a Jasco J-715 spectropolarimeter (Tokyo, Japan) using a quartz cell of 1 mm path length between 195 and 250 nm at room temperature. The concentration of peptides was 120 μg/mL in 10 mM Tris buffer (pH 7.4) containing 25 mM SDS. Two scans with a scan speed of 10 nm/min were averaged for each peptide. CD spectra were expressed as the mean residue ellipticity. The α helicity of the peptide was determined from the mean residue ellipticity $[\theta]$ at 222 nm according to the relationship $[\theta]_{222} = -30300$ $[\alpha] - 2340$ (where $[\alpha]$ is the amount of helix).[41]

4.6. Antimicrobial assay

A detailed in vitro antimicrobial assay has been described elsewhere.[42] The in vitro antimicrobial assay was done using a modified microdilution technique in a 96-well microplate. Antibiotic medium 3 (M3; pH 7.0 at 25 °C, Difco) was used as the antibacterial assay media. Freshly grown cells on antibiotic medium 3 agar plate were suspended in physiological saline to 10^4 cells per 1 ml of 2× concentrated medium and used as the inoculum. Just before the assay, solid samples were dissolved, added to the wells (100 μl per well) and the wells were serially diluted twofold. After inoculation (100 μl/well, 1×10^4 cells/ml), plates were incubated at 37 °C for 24 h, and the absorbance at 620 nm was measured by an ELISA reader (Spectra, Austria) to assess cell growth. The antifungal assays were done in Sabraud-2% dextrose broth (SB; pH 5.6 at 25 °C, Merck) and the plates were incubated at 30 °C for 24 h. The minimum inhibitory concentration (MIC) was defined as the concentration at which 100% inhibition was observed. All MICs were determined from two independent experiments performed in duplicate.

4.7. Hemolytic activity

The detailed method has been described elsewhere.[43] Packed human erythrocytes were washed three times with buffer (150 mM KCl, 5 mM Tris–HCl, pH 7.4) then suspended in ten volume of the same buffer (stock cell suspension). For antibiotic treatment, the cell stock suspension was 25-fold diluted with the same buffer and preincubated at 37 °C in a water bath for 15 min. The final concentration of erythrocytes was approximately 0.4% (v/v). Increasing amounts of the test samples (1.56–200 μg/mL) were then added. After incubation for 1 h at 37 °C, samples were centrifuged at 4000*g* for 5 min and the absorbance of supernatant was determined at 540 nm. Hemolysis effected by 0.1% Triton X-100 was considered as 100% and the well-known hemolytic peptide, melittin was used as a reference compound in this assay.

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